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研究課題名（和文）Molecular characterization, its mutation pattern and the molecular surveillance of SARS-CoV-2 in Hiroshima

研究課題名（英文）Molecular characterization, its mutation pattern and the molecular surveillance of SARS-CoV-2 in Hiroshima

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研究成果の概要（和文）：本研究では、734の唾液検体を用いて、Sanger法による部分スパイクシーケンスによるSARS-CoV-2変異株分類の開発を行ったところ、99.6%の増幅と96.9%のゲノムデータの検出が可能であった。これは、Sanger法が大量の検体の変異株スクリーニングにについて、精度が高く有効であることを示した。このことから、Sanger法は、次世代シーケンサー（NGS）が容易に利用できない地域において、低コストで時間効率の高い、実用的で普遍的な適用可能となり得る。既存の変異体の同定だけでなく、希少な変異や新たな変異の検査も可能であり、パンデミックの予防と制御の取り組みにおいて非常に有用と考えられた。

研究成果の学術的意義や社会的意義
This study introduced the possibility of SARS-CoV-2 variant screening by partial sequencing at spike region which has higher screening rate then next generation sequencing and applicable to the resource limited setting for molecular surveillance and pandemic control.

研究成果の概要（英文）：A study on development of Sanger based SARS-CoV-2 variants classification by partial spike sequencing among 734 stocked samples provided 99.6% successful amplification and 96.9% genomic data which allowed for variant identification and molecular surveillance of SARS-CoV-2 variants in Hiroshima. The findings of this study demonstrate that Sanger sequencing is a reliable and effective approach for screening a large number of samples to detect significant SARS-CoV-2 variants. In comparison to Next Generation Sequencing (NGS), our method offers a practical and universally applicable tool that is both low-cost and time-efficient, particularly in regions where NGS is not readily accessible. Our method enables the identification not only of existing variants but also the examination of uncommon mutations or newly emerged variants, making it invaluable for pandemic prevention and control efforts.

研究分野：Infectious Disease Control

キーワード：SARS-CoV-2　Surveillance　Infection control

１．研究開始当初の背景

COVID-19 is a respiratory illness caused by a virus called SARS-CoV-2. It originated from animals and is closely related to a bat coronavirus [1, 2]. The pandemic started in late 2019 in Wuhan, China [3, 4], and has since spread worldwide, with millions of cases and deaths reported. The virus has mutated over time, giving rise to different variants, some of which are more transmissible and severe. Notable variants include the Alpha, Beta, and Gamma variants[5, 6]. Hiroshima, a city in Japan, has experienced several waves of COVID-19 outbreaks. The fourth wave has been particularly severe, and the research aims to understand if it is caused by new mutant variants or imported cases. The ultimate goal is to propose effective strategies for the prevention and control of the disease.

The COVID-19 pandemic continues to impact global populations, causing millions of deaths and disrupting various aspects of society, including the economy and social conditions. Despite the development and distribution of vaccines against SARS-CoV-2, the emergence of numerous mutant variants poses a significant challenge to controlling the pandemic. This study specifically emphasizes understanding the molecular characteristics, mutation patterns, and developing an effective screening methodology for identifying mutant variants.

２．研究の目的

This study aims to analyze the molecular characteristics and mutation patterns of SARS-CoV-2 during the third and fourth waves in Hiroshima. This study intends to develop a screening method to identify mutations in the spike region of the virus, which is crucial for its entry into host cells. This study will involve analyzing the virus's genetic material using Next Generation Sequencing (NGS)[7] and developing a more efficient screening method.

３．研究の方法

3.1 Study subjects:

A total of 734 samples were included in this study, collected between September 1, 2020, and May 25, 2021, from confirmed COVID-19 cases in various cities within Hiroshima prefecture. The samples consisted of 287 nasopharyngeal swabs and 447 saliva samples. The flow of study subjects is depicted in Fig. 1, and some samples were provided by the Hiroshima City Institute of Public Health, while others were collected from three hospitals: Hiroshima University Hospital, Funairi Hospital, and Hiroshima Prefectural Hospital. These hospitals were among the five main COVID-19 treatment centers in Hiroshima Prefecture.

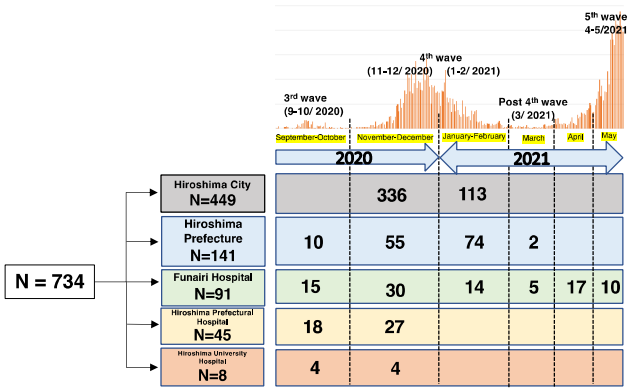


Figure 1. Flow of study subjects in Hiroshima, Japan

3.2 Nucleic acid extraction and quantification of SARS-CoV-2:

Fifty microliters of each sample from COVID-19 confirmed cases underwent nucleic acid extraction using SMITEST EX-R&D (MBL, USA). The extracted template RNA was quantitatively measured for viral titer using specific primers (NIID_2019-n-CoV-N-F2 and NIID_2019-nCoV-N-R2) and a probe (NIID_2019-nCoV-N-P2) targeting the nucleocapsid (N) protein. Real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was employed

for this analysis, with a standard template of known concentration and negative controls. The measured viral titers were then converted into the number of viral copies per milliliter.

3.3 Standard protocol for spike region amplification by nested RT-PCR:

To amplify the spike protein of SARS-CoV-2, 5% (2.5 µL) of the template RNA was used in a nested reverse transcriptase polymerase chain reaction (RT-PCR). The first round of nested RT-PCR was carried out using the PrimeScript One-Step RT-PCR kit Ver.2, while the second round was performed using TaKaRa Ex Taq Hot Start version. Gel electrophoresis was used to examine the amplified products.

3.4 Volume-up protocol for previously negative samples:

If the nested RT-PCR yielded negative results in the first attempt, the samples underwent nucleic acid extraction from the original samples using SMI-TEST. The extracted template RNA was then subjected to a volume-up reaction by repeating the first round nested RT-PCR.

3.5 In-house developed primer sets for Sanger sequencing:

Three different primer sets were designed for variant screening by Sanger sequencing. These sets, named hCoV-Spike-A, hCoV-Spike-B, and hCoV-Spike-C, targeted specific regions of the spike protein to identify different SARS-CoV-2 variants. The primer sets covered various nucleotide positions and were used to classify the variants based on predetermined criteria (Fig. 2).

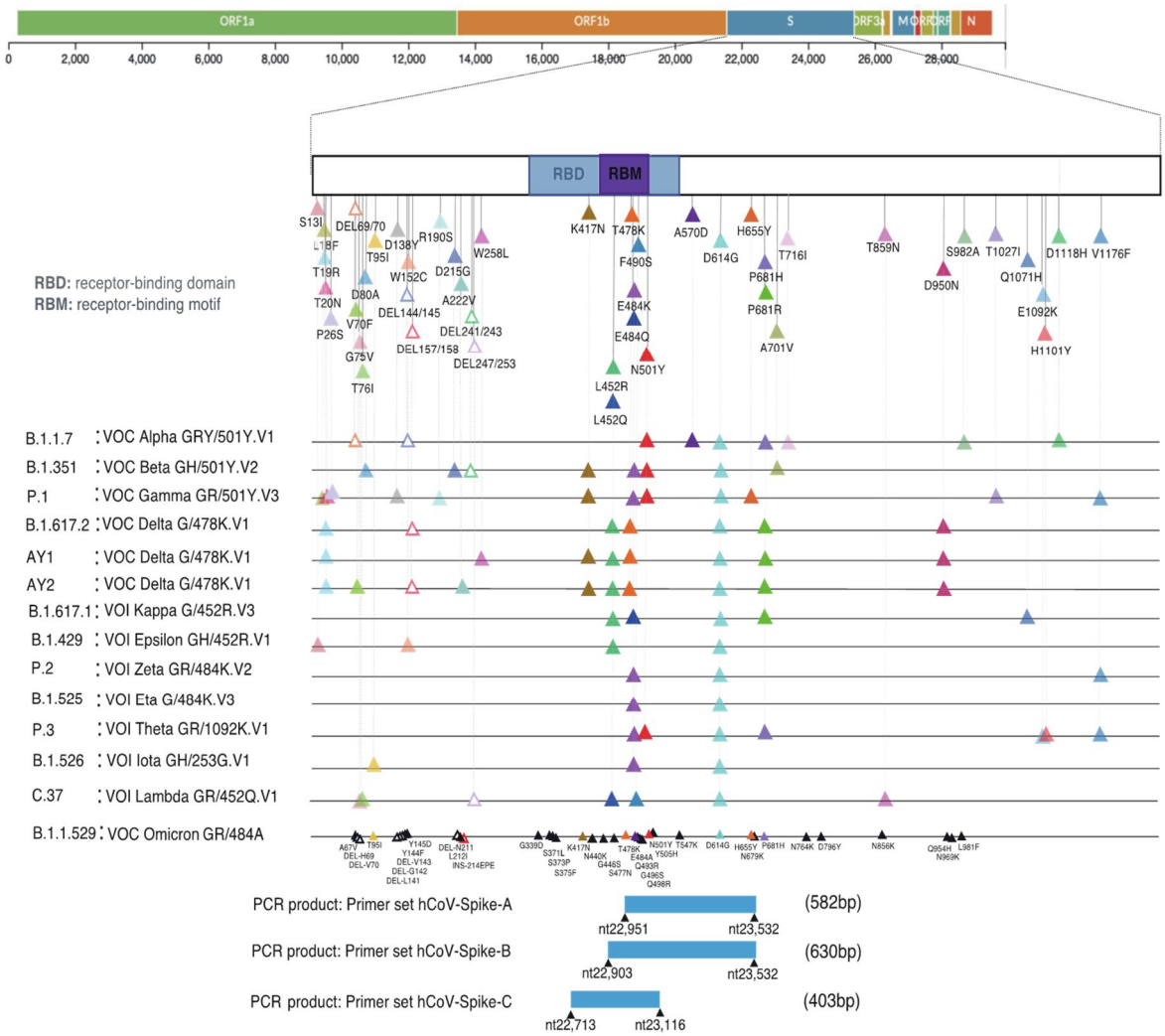


Figure-2: Schematic mutation pattern of notable SARS-COV-2 variants and its primer product

3.6 Sanger sequencing of SARS-CoV-2 spike protein partial genomes:

Positive nested RT-PCR products were subjected to Sanger sequencing to obtain partial genomes of the targeted spike region. This sequencing was performed using a 3730xl DNA sequencer and the BigDye Terminator v3.1 Cycle Sequencing Kit. Corresponding primer sets were used for the sequencing process.

3.7 Validation of Sanger sequencing strategy:

To validate the absence of amplification-induced mutations during the 70 amplification cycles, eight previously submitted SARS-CoV-2 isolates from Hiroshima were subjected to the standard protocol for nested RT-PCR and in-house developed primer sets. Quadruplicate tests were conducted for each isolate, totaling 32 tests. The amplified products obtained through Sanger sequencing were compared to the reference sequences obtained through next-generation sequencing (NGS), and a 100% identity was observed, confirming the absence of amplification-induced mutations and validating the agreement between Sanger sequencing and NGS

4．研究成果

This study included a total of 734 samples collected from confirmed COVID-19 cases in Hiroshima Prefecture between September 1, 2020, and May 15, 2021. The viral titers of these samples ranged from 1×10^1 to 4.21×10^8 copies/mL.

4.1 Nested RT-PCR positive rate:

When using the primer set hCoV-Spike-A, all samples underwent RT-PCR, and 93 out of the total 734 samples showed negative amplification according to the standard protocol. This resulted in a positive rate of 87.3% for nested RT-PCR. Most of the samples with negative nested RT-PCR had a low viral titer below 103 copies/mL. After a second attempt of nested RT-PCR using the volume-up protocol, only three samples remained negative, resulting in an increased positive amplification rate of 99.6% after the volume-up reaction. Similarly, the positive amplification rate using the primer set hCoV-Spike-B with the standard protocol was 83.3% (16 out of 96 were negative), which increased to 95.8% after the volume-up reaction. Furthermore, the positive amplification rate using the primer set hCoV-Spike-C in the standard protocol was 93.8% (6 out of 96 were negative), and it increased to 96.9% after the volume-up reaction (Fig. 3).

4.2 Readiness of targeted partial genomes among positive amplified products:

Among the 730 nested RT-PCR positive samples that underwent Sanger Sequencing using the primer set hCoV-Spike-A, 23 isolates could not be analyzed due to the presence of many unidentified "N" in the sequences. Therefore, the readiness of the sequences using the primer set hCoV-Spike-A was 96.9%, while both the respective 92 and 93 amplified products using primer set hCoV-Spike-B and hCoV-Spike-C provided 100% readiness by Sanger Sequencing (Fig. 3).

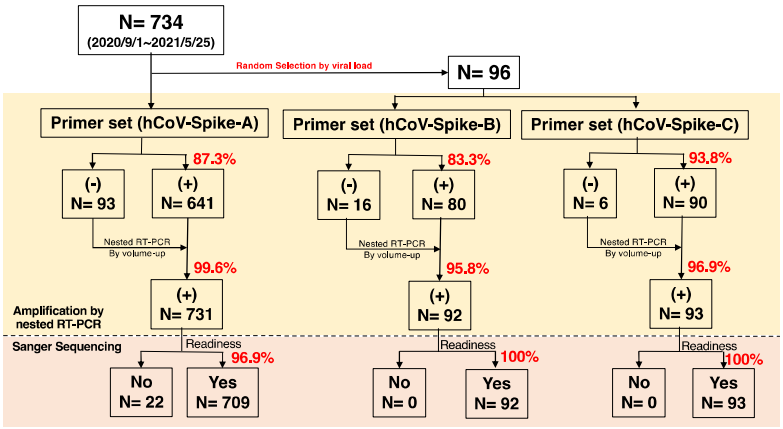
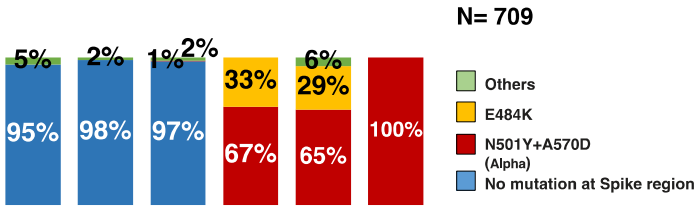


Figure-3: Amplification positive rate and readiness of direct sequences using primer set hCoV-Spike-A,hCoV-Spike-B and hCoV-Spike-C

4.3 Distribution of notable SARS-CoV-2 variants through screening:

Out of the 709 isolates screened, 48 isolates had specific mutations as shown in Figs. 5 and 6, resulting in an overall mutation rate of 6.8%. SARS-CoV-2 variants were found in 5%, 2%, and 3% respectively during the periods of September-October, November-December 2020, and January-February 2021. In March 2021, 67% of the variants were identified as B.1.1.7 (Alpha), while the remaining variants had a single E484K mutation. In April 2021, 65%, 29%, and 6% of the variants were B.1.1.7 (Alpha), E484K mutations, and other forms of mutations,

respectively. In May 2021, all 10 isolates were identified as B.1.1.7 (Alpha). Other forms of mutations included single mutations at N501S, I584V, R481K, F515L, P521L, T553S, N606S, A609G, double mutation at K557E and Q613R, and triple mutation at L513F, Q580R, and V615A (Fig. 4).



	FY 2020		FY 2021				Total
	Sept-Oct	Nov-Dec	Jan-Feb	March	April	May	
Number screened	42	444	190	6	17	10	709
No mutation at Spike region	40	436	185	0	0	0	661
N501Y+A570D (Alpha)	0	0	1	4	11	10	26
E484K	0	0	0	2	5	0	7
Others*	2	8	4	0	1	0	15

* N501S, I584V, R481K, F515L, P521L, T553S, N606S, A609G, K557E, Q613R, L513F, Q580R, V615A.

Figure-4: Prevalence of the notable SARS-CoV-2 mutant variants in Hiroshima

5. References

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５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　1件）

１．著者名 Ko K, Takahashi K, Nagashima S, E B, Ouoba S, Hussain MRA, Akita T, Sugiyama A, Sakaguchi T, Tahara H, Ohge H, Ohdan H, Kubo T, Ishikawa N, Takafuta T, Fujii Y, Mimori M, Okada F, Kishita E, Ariyoshi K, Kuwabara M, Tanaka J	４．巻 12
２．論文標題 Mass Screening of SARS-CoV-2 Variants using Sanger Sequencing Strategy in Hiroshima, Japan	５．発行年 2022年
３．雑誌名 Scientific Reports	６．最初と最後の頁 2419
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41598-022-04952-2	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関